REMARKS

Status of the claims

Claims 1-36 are pending and claims 1-20 are under consideration in this application, claims 21-36 having been withdrawn for allegedly being drawn to a separate invention. All the claims under consideration stand rejected.

In order to retain the right under 37 C.F.R. § 1.121 to rejoin claims directed to methods of using the presently claimed polypeptides, polypeptide segments, and compositions once the present claims have been held allowable, appropriate method of use claims have been amended herein to conform them to the scope of the claims presently under consideration. "Method of use" claims that have been so amended include claims 21-28.

None of the amendments made herein or the new claims added herein add new matter.

After entry of the amendments made herein claims 1-36 will be pending and claims 1-20 will be under consideration in this application.

Objections to the specification

In response to the comments on page 2, lines 9-22, of the Office Action, Applicants have replaced the referral to U.S. Patent Application Serial No. 08/796,792 on page 1, line 16, of the specification with a referral to the appropriate U.S. Patent No., i.e., U.S. Patent No. 6,087,163.

In response to the comments on page 2, lines 22-26, of the Office Action, Applicants have deleted several hyperlinks from the specification on pages 13 and 14 of the specification.

In response to the comment on page 3, lines 1-3, of the Office Action, Applicants have inserted the word "of" before the term "mtsp" in the title of Table 4, on page 20 of the specification.

Applicants respectfully submit that the objections to the specification on pages 2 and 3 of the Office Action are moot in view of the amendments to the specification.

35 U.S.C. § 112, first paragraph, rejections

(a) Claims 9 and 10 stand rejected on the grounds that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

From the comments on page 3, line 13, to page 4, line 13, of the Office Action, Applicants understand the Examiner's position to be that use of the DNA molecules of the invention as vaccines is not enabled by the specification in view of the state of the art at the time the application was filed. While not agreeing with this position, in order to expedite prosecution of the application, Applicants have cancelled claims 9 and 10 without prejudice to their being pursued in a separate application.

Applicants respectfully submit that the rejection is moot in light of the above amendment.

(b) Claims 17-20 stand rejected on the grounds that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

From the comments on page 4, line 14, to page 5, line 21, of the Office Action, Applicants understand the Examiner's position to be that diagnosis of tuberculosis using the polypeptides of the invention is not enabled by the specification in view of the state of the art at the time the application was filed. Applicants respectfully disagree with this position and submit that one skilled in the art would have expected that at least a substantial number of the *Mycobacteria tuberculosis* polypeptides identified by the analyses described in the Example on pages 13-22 of the application as being secreted polypeptides would be useful for diagnosis of tuberculosis.

A scientific article from the laboratory of Dr. Maria Laura Gennaro, one of the Applicants, published after the priority date of the instant application shows that such an artisan would have been correct. The article demonstrates that at least five of these polypeptides, alone or in combination with other polypeptides of the invention or a "prior art" polypeptide, to be useful as diagnostic agents [Amor et al. (2005) Scandinavian J. of Immunol. 61:139-146; copy

enclosed as Exhibit A]. Thus, the data presented in the article show that levels of antibodies to these five polypeptides above a threshold level (established in control patients) could be detected in a significant fraction of tuberculosis patients while either no, or much fewer, control patients had levels of such antibodies above this threshold level (see Fig. 4 and accompanying text in the second paragraph of column two on page 144). The relevant polypeptides are MTSP1, MTSP21, MTSP23, MTSP36, and MTSP43 (designated Rv0603, Rv1804c, Rv1271c, Rv2253, and Rv0203, respectively, in Amor et al.). Claims 17-21 have been amended to specify only these five polypeptides, or functional segments of them. Applicants retain the right to pursue similar claims specifying one or more of the deleted polypeptides, or functional segments thereof, in a separate application.

In light of the above considerations, Applicants respectfully request that the rejections under 35 U.S.C. § 112, first paragraph, be withdrawn.

35 U.S.C. § 112, second paragraph, rejection

Claims 1-20 stand rejected as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter that the Applicant regards as the invention.

From the comments on page 6, lines 4-11 and 16-18, of the Office Action, Applicants understand the Examiner's position to be that one skilled in the art would be not be apprised by the specification as to how many conservative substitutions the polypeptides could tolerate without losing their *Mycobacteria tuberculosis* specific antigenic and immunogenic properties. Applicants respectfully disagree with this position in that those skilled in the art are entirely familiar with methods for assessing protein antigenicity and immunogenicity, as the terms are used herein (see, for example, page 7, line 3, to page 8, line 12, of the instant specification). Nevertheless, in order to expedite prosecution of the instant application, Applicants have deleted embodiments specifying variants containing conservative substitutions from the claims. Applicants retain the right to pursue claims specifying polypeptides containing conservative substitutions in a separate application.

From the comments on page 6, lines 11-16, of the Office Action, Applicants understand the Examiner's position to be that the specification does not indicate which of the polypeptides disclosed by the instant application would have *Mycobacteria tuberculosis* specific antigenic and immunogenic properties. Applicants respectfully disagree with this position and submit that one skilled in the art, at the priority date of the instant application, would have expected that a substantial number of the polypeptides would have such properties and would certainly have known how to test for them. The scientific article cited above (Amor et al.) confirms that such an artisan would have been correct. From the experiments described on page 143, the first paragraph on page 144, and the accompanying data depicted in Table 2 and Fig. 2 in Amor et al., the authors of the article concluded that six polypeptides were specifically expressed by *Mycobacteria tuberculosis* complex bacteria. By definition, these polypeptides would have *Mycobacteria tuberculosis*-specific antigenic and immunogenic properties. The relevant polypeptides are MTSP15, MTSP21, MTSP25, MTSP36, MTSP43, and MTSP47 (designated Rv0617, Rv1804c, Rv2389c, Rv2253, Rv0203, and RV2290, respectively, in Amor et al.). Claims 1 and 11 have been amended to specify only these six polypeptides. Applicants retain the right to pursue similar claims specifying one or more of the deleted polypeptides, or functional segments thereof, in a separate application.

Interestingly, while MTSP23 (Rv1271c) and MTSP1 (Rv0603) were not classified in Amor et al. as being specific to the *Mycobacteria tuberculosis* complex (see Table 2), because their non-*Mycobacteria tuberculosis* complex expression was only in mycobacterial strains of little medical significance (*Mycobacteria malmoensa* and/or *Mycobacteria kanasii*; page 143, column 2, paragraph 2), they were included in the study described above that confirmed their usefulness in diagnostic assays.

In light of the above considerations, Applicants respectfully request that the rejections under 35 U.S.C. § 112, second paragraph, be withdrawn.

CONCLUSIONS

Applicant submits that the pending claims patentably define the invention and request that the Examiner permit the pending claims to pass to allowance.

If the Examiner would like to discuss any of the issues raised in the Office Action, Applicant's undersigned representative can be reached at the telephone number listed below.

Enclosed is a request for an automatic extension of time, and a check in payment of the extension in time. Please apply any other charges or credits to Deposit Account No. 06-1050, referencing Attorney Docket No. 07763-042001.

Respectfully submitted,

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Immunological Characterization of Novel Secreted Antigens of *Mycobacterium tuberculosis*

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Abstract

Proteins secreted by *Mycobacterium tuberculosis* are targets of host immune responses and as such are investigated for vaccine and immunodiagnostics development. Computer-driven searches of the *M. tuberculosis* H_{37}Rv genome had previously identified 45 novel secreted proteins. Here, we report the characterization of these antigens in terms of specificity for the *M. tuberculosis* complex and the ability to induce human immune responses. BLAST homology searches and Southern hybridization identified 10 genes that were either specific for the *M. tuberculosis* complex or found in only two nontuberculous mycobacterial species of minor medical significance. Selected recombinant proteins were purified from *Escherichia coli* cells and tested for the ability to elicit antibody responses in tuberculosis patients. Reactivity of the serum panel was ' 36% with at least one of five novel proteins (Rv0203, Rv0603, Rv1271c, Rv1804c and Rv2253), 56% with the 38 kDa lipoprotein, a *M. tuberculosis* antigen known to be highly seroreactive, and 68% with a combination of Rv0203, Rv1271c and the 38 kDa antigen. Thus, at least five novel secreted proteins induce antibody responses during active disease; some of these proteins may increase the sensitivity of serological assays based on the 38 kDa antigen.

Introduction

Proteins released into the extracellular milieu by *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), have been the focus of research aimed at developing TB vaccines and immunodiagnostics, because they are thought to induce protective immunity and immune responses of diagnostic value [1–5]. While our knowledge of protein secretion in *M. tuberculosis* is still limited, it is clear that this bacterium possesses a type II, *sec*-dependent pathway that mediates export of proteins carrying NH_2-terminal, secretion of signal peptides [6, 7]. Conserved features of signal peptides has made it possible to use bioinformatics to search the *M. tuberculosis* genome for proteins targeted for *sec*-dependent secretion [8–11]. The approach taken by our laboratory entailed a genome-wide screen utilizing four different computer programs to identify the proteins that carry NH_2-terminal, cleavable signal peptides but lack membrane-spanning domains and other membrane-anchoring motifs in the putative mature protein ([10]; http://www.tbsp.phri.org). Fifty-two proteins were identified, of which 45 had not been previously reported (Fig. 1). In the present work, we screened the newly identified secreted protein pool for proteins that elicit immune responses of diagnostic value.

Forty-five genes encoding secreted proteins of *M. tuberculosis* were characterized for distribution among tuberculous and nontuberculous mycobacteria. Selected proteins were examined for reactivity with sera from patients having pulmonary TB and control patients having lung pathology other than TB. Five novel proteins were found to elicit measurable, albeit modest, antibody responses during active disease in humans. Some of these proteins may have serodiagnostic value.

Materials and methods

Mycobacterial strains. DNA was isolated from tuberculous and nontuberculous mycobacteria: *M. tuberculosis* strain W (PHRI Collection), *M. tuberculosis* H_{37}Rv ATCC 25618, *Mycobacterium bovis* (PHRI Collection), *M. bovis* Bacille

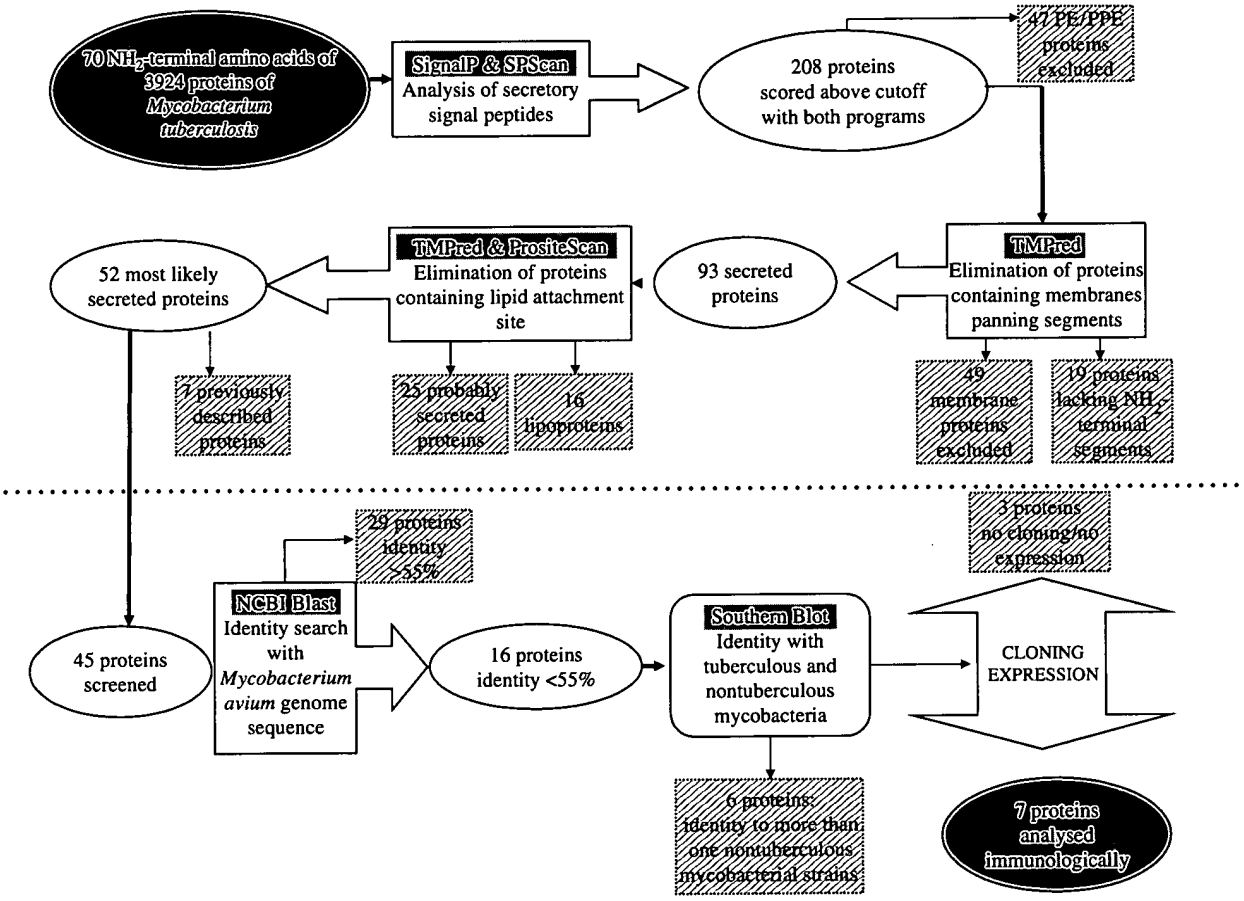

Figure 1 Flowchart of the experimental work. The flowchart illustrates the strategy used for selecting secreted proteins of *Mycobacterium tuberculosis* for serological analysis. The top part of the flowchart (above the dotted line) summarizes a previous, computer-driven search [10] that utilized four computer programs (SIGNALP, SPSCAN, TMPRED and PROSITESCAN) to identify *M. tuberculosis* proteins that were translocated across the cell envelope and released into the external milieu. Selected proteins carried NH_2-terminal, cleavable secretory signal peptides but lacked membrane-spanning domains and other membrane-anchoring motifs in the putative mature proteins. Forty-five had not previously been reported as secreted proteins in original articles (of these, three – Rv1566c, Rv3036c and Rv2253 – were also identified as secreted proteins in an independent study [31]). In the present study (bottom part of the flowchart), amino acid sequences deduced from the selected 45 proteins were further analysed with a BLAST homology program for homologues with *Mycobacterium avium* proteins: proteins of *M. tuberculosis* showing a >55% identity with *M. avium* proteins were not further studied. Subsequent Southern blot analysis with DNA from tuberculous and nontuberculous mycobacteria identified 10 *M. tuberculosis*-complex-specific proteins that were characterized for their ability to induce antibody responses during human tuberculosis. Shaded boxes show the number of proteins eliminated at each step of the selection process.

Calmette-Guerin (BCG) substrain Japan ATCC 35737, *Mycobacterium africanum* ATCC 35711, *Mycobacterium avium* ATCC 25291, *Mycobacterium chelonae* ATCC 35752, *Mycobacterium diernhoferi* ATCC 19340, *Mycobacterium fortuitum* TMC 1530, *Mycobacterium gastri* ATCC 15754, *Mycobacterium gordonae* ATCC 14470, *Mycobacterium haemophilum* (PHRI Collection), *Mycobacterium intracellulare* ATCC 13950, *Mycobacterium kansasii* ATCC 12478 and ATCC 35775, *Mycobacterium lufu* (PHRI Collection), *Mycobacterium malmoense* ATCC 29571, *Mycobacterium marinum* ATCC 927, *Mycobacterium microti* ATCC 35782, *Mycobacterium phlei* ATCC 11758, *Mycobacterium scrofulaceum* ATCC 35785, *Mycobacterium ulcerans* ATCC 35840, *Mycobacterium vaccae* ATCC 15483 and *Mycobacterium xenopi* ATCC 19250.

Bacteriological methods and DNA hybridization. Mycobacterial strains were cultured at 37 °C on Lowenstein–Jensen slants for 4 days (fast-growing strains) or 6 weeks (slow-growing strains). An exception was *M. haemophilum* which was cultured on chocolate agar plates at 30 °C. Chromosomal DNA was isolated according to standard procedures [12], digested with PvuII and electrophoresed on 1% agarose gels. The separated digestion products were transferred to Hybond N^+ membranes (Amersham Biosciences AB, Uppsala, Sweden) by use of a VacuGene XL blotting system (Amersham Biosciences AB). Membrane-bound DNA was hybridized overnight with a labelled DNA probe specific for each of the selected *M. tuberculosis* genes. Hybridized bands were visualized by enhanced chemoluminescence (ECL) detection kit

(Amersham Biosciences AB). All procedures were performed according to the manufacturer's instructions.

Antigens. M. tuberculosis genes were amplified by polymerase chain reaction (PCR) using specific primers. Amplification products were purified by excision from 1% agarose gels and cloned into the shuttle vector PCR Blunt II (Promega U.S., Madison, WI, USA), as described in [13]. Appropriate restriction fragments were excised using B*am*HI and H*ind*III restriction endonucleases (New England Biolabs, Beverly, MA, USA), purified and cloned into pQE30 (Qiagen Inc., Valencia, CA, USA), an expression plasmid vector of *Escherichia coli*. Genes encoding small proteins (<15 kDa, predicted molecular weight), such as Rv1271c and Rv1804c, were also cloned into pET102 (Invitrogen Corporation, Carlsbad, CA, USA), an expression vector designed to produce recombinant proteins fused to the 11.5 kDa thioredoxin of *E. coli*. This protocol should help minimize degradation of small proteins [14].

Recombinant proteins were expressed either as NH_2-terminally (pQE30) or COOH-terminally (pET102) polyhistidine-tagged fusion proteins and purified from *E. coli* XL1 blue cells by sequential chromatography with metal chelate affinity, size exclusion and anion exchange columns [15] to near-homogeneity (>95%), as determined by Coomassie Blue staining of protein separated by denaturing polyacrylamide gel electrophoresis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. Purified proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 16% polyacrylamide gels [16] and transferred to immunoblot membranes (Bio-Rad Laboratories, Hercules, CA, USA) according to Gershoni and coworkers [17]. Membranes were incubated in phosphate-buffered saline (PBS) with 3% nonfat skim milk (Bio-Rad Laboratories) for 1 h at 37 °C with an antihistidine mouse monoclonal immunoglobulin (Ig)G antibody (Amersham Biosciences AB) diluted 1:2000. Bound antibodies were revealed by incubating membranes in PBS with 3% nonfat skim milk for 1 h at 37 °C with horseradish-peroxidase-coupled rabbit antimouse antibody (Bio-Rad Laboratories) at a 1:2000 dilution. Detection was performed using the AEC staining kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's recommendation.

Table 1 List of 29 genes encoding secreted proteins of *Mycobacterium tuberculosis* showing a >55% identity with *Mycobacterium avium* proteins

Gene number	Gene name/Proposed function
Rv0199	Putative conserved membrane protein*
Rv0309	Heavy metal-associated domain
Rv0315	Glucanase
Rv0360	Conserved hypothetical protein
Rv0398c	Possible secreted protein
Rv0455c	Conserved hypothetical protein
Rv0477	Putative conserved secreted protein
Rv0559c	Putative conserved secreted protein
Rv0592	*mce2D*/mammalian cell entry-family protein involved in host cell invasion
Rv0674	Conserved hypothetical protein
Rv0867c	*rpfA*/May promote resuscitation/growth of dormant cells
Rv1006	Possible secreted protein
Rv1269c	Conserved probable secreted protein
Rv1488	Hemopexin domain signature
Rv1566c	Putative invasion protein
Rv1891	Putative exported conserved protein
Rv1906c	Conserved hypothetical protein
Rv1974	Probable conserved membrane protein*
Rv2223c	Putative exported protease
Rv2450c	*rpfE*/May promote resuscitation/growth of dormant cells
Rv3013	Conserved hypothetical protein
Rv3036c	*TB22.2*/Probable conserved secreted protein
Rv3096	Glycosylhydrolase family 5
Rv3106	*fprA*/Ferredoxin reductase
Rv3170	*aofH*/Probable flavin-containing monoamine oxidase
Rv3207c	Zn protease
Rv3354	Conserved hypothetical protein
Rv3572	Possible secreted protein
Rv3668c	Possible protease

The gene numbering is according to Cole *et al.* [18]. Gene function was as reported in TubercuList (http://www.genolist.pasteur.fr/Tuberculist).
*The annotation as putative membrane proteins reported in Tuberculist for two gene products in this table (and for Rv2576c in Table 2) can be attributed to a predicted transmembrane domain in the NH_2-terminal portion of the deduced amino acid sequence. That transmembrane domain is consistent with our predicted signal peptide (http://www.tbsp.phri.org).

Sera. Serum samples were obtained from 50 TB patients and 48 negative control patients. Patients were diagnosed in Nuevo Leon, in Mexico, with smear positive ($n = 46$) or smear negative ($n = 4$) pulmonary TB. Blood samples were collected on the day of admission to the healthcare facility, prior to anti-TB chemotherapy. Negative control sera were obtained from 48 patients from the same hospital diagnosed with pulmonary disease other than TB (e.g. pneumonia, bronchiectasis, pulmonary fibrosis or lung cancer). For the control patients, information about recent exposure to active TB cases or history of past TB was not always conclusive. A positive history of vaccination with *M. bovis* BCG was ascertained for 32/50 TB patients and 21/48 control patients with disease other than TB. The remaining subjects were either non-BCG vaccinated (10/50 TB patients and 14/48 non-TB control patients) or BCG vaccination status was unknown. The study population was negative to infection with human immunodeficiency virus (HIV).

Enzyme-linked immunosorbent assay. Polystyrene 96-well microtitre plates (Bio-Rad Laboratories) were coated overnight with purified *M. tuberculosis* protein at 1.0–2.0 μg/ml (0.2 ml per well) in carbonate–bicarbonate buffer (pH 9.6). Plates were blocked with 1% nonfat skim milk (Sigma-Aldrich) in PBS (pH 7.4) containing 0.05% Tween 20 (PBS-T) for 2 h at 37 °C and washed two times with PBS-T. Serum samples were diluted 1:50 in 1% nonfat skim milk in PBS-T, and 0.2 ml of diluted serum was added to antigen-coated wells and incubated for 30 min at 37 °C. Plates were washed five times with PBS-T and then incubated for 30 min with 0.2 ml of goat antihuman IgG labelled with horseradish peroxidase (Dako, Glostrup, Denmark) diluted at 1:20,000 in 1% nonfat skim milk in PBS-T. Plates were washed five times with PBS-T, and 0.2 ml of tetramethylbenzidine (TMB) substrate (Bio-Rad Laboratories) was added to each well. After the addition of 0.05 ml of 1 N H_2SO_4 to stop the reaction, the optical density at 450 nm (OD_{450}) was measured with an automatic microplate reader (Spectra Shell; Tecan Systems Inc., San Jose, CA, USA).

Serologic data analysis. Antibody responses were evaluated by calculating, for each antigen, cutoff values as the means of OD_{450} values obtained with 48 negative control sera plus three SD. Comparisons of patient and control population were conducted by *t*-test: differences in specific antibody levels between patient and control populations

Table 2 Characterization by Southern blot analysis of 16 genes of *Mycobacterium tuberculosis* showing <55% identity with *Mycobacterium avium* proteins

		Nontuberculous Mycobacteria	
Gene number	*M. tuberculosis* complex	Strong signal	Weak signal
Rv0203*	Specific	–	–
Rv0603*	Nonspecific	*Mycobacterium kansasii*	–
Rv0617	Specific	–	–
Rv1242	Nonspecific	*M. kansasii*	–
Rv1268c	Nonspecific	*Mycobacterium fortuitum*	*M. kansasii* *Mycobacterium malmoense*
Rv1271c*	Nonspecific	*M. malmoense*	*M. kansasii*
Rv1291c	Nonspecific	*Mycobacterium scrofulaceum* *Mycobacterium marinum* *M. malmoense* *Mycobacterium gastri*	*M. fortuitum* *Mycobacterium chelonae* *Mycobacterium ulcerans*
Rv1352	Nonspecific	*M. kansasii* *M malmoense*	*Mycobacterium xenopi* *M. gastri*
Rv1419	Nonspecific	*M. malmoense* *Mycobacterium haemophilum* *M. kansasii*	*M. fortuitum* *M. marinum* *M. xenopi*
Rv1804c*	Specific	–	–
Rv1813c*	Nonspecific	–	*M. kansasii* *M. scrofulaceum*
Rv2253*	Specific	–	–
Rv2290 (*lppO*)*	Specific	–	–
Rv2389c (*rpfD*)	Specific	–	–
Rv2576c	Nonspecific	*M. kansasii*	*M. fortuitum* *M. haemophilum* *Mycobacterium intracellulare* *M. malmoense* *M. marinum*
Rv3333c	Nonspecific	*M. gastri*	*M. kansasii* *M. avium*

*Genes that were cloned, expressed, purified and characterized serologically in this work.

were examined using two-sided *t*-tests and a significance level of 0.05.

Results and discussion

Gene selection

In our previous work [10], the 3924 proteins deduced from the *M. tuberculosis* H_{37}Rv genome [18] were analysed by existing computer programs to identify proteins that were likely to be secreted by *M. tuberculosis* into the extracellular medium. Gene selection by the bioinformatic approach was validated in a gene subset using *E. coli phoA'* fusion technology. Forty-five had not previously been reported as secreted proteins in original articles. We screened the 45 proteins for the presence of homologues in *M. avium*, a common nontuberculous mycobacterial species associated with human infection, because homology with *M. avium* proteins makes *M. tuberculosis* antigens unsuitable for TB immunodiagnosis. For this analysis, we used the *M. avium* genome sequence (http://www.tigr.org) [19] and a BLAST protein homology program; amino acid sequence identity of 55% was set as an arbitrary cutoff. Twenty-nine genes, which were classified as having high homology (>55% identity), were not further considered (Table 1).

Sixteen *M. tuberculosis* genes that had low homology (<55% identity) with *M. avium* proteins (Table 2) were characterized in terms of distribution among mycobacterial species by Southern hybridization. Southern blot analysis was performed by hybridizing *PvuII*-digested DNA from five tuberculous and 18 nontuberculous mycobacteria with gene-specific DNA fragments (*Materials and methods*). Based on DNA hybridization signals, six genes were classified as specific for the *M. tuberculosis* complex (Table 2) and selected for expression and purification of recombinant protein. Of the remaining 10 genes that were not specific for the *M. tuberculosis* complex, four (Rv0603, Rv1242, Rv1271c and Rv1813c) were also selected for protein production, because these gave strong hybridization signals with either *M. kansasii* or *M. malmoense* (Table 2), which are of relatively little medical significance (*M. malmoense* is found more commonly in some northern European countries [20]; *M. kansasii*, which is rare in the general population, is detected almost exclusively in HIV-infected individuals [21–23]). In the examples of Southern blot patterns shown in Fig. 2, Rv1271c hybridized with the *M. tuberculosis* complex as well as with

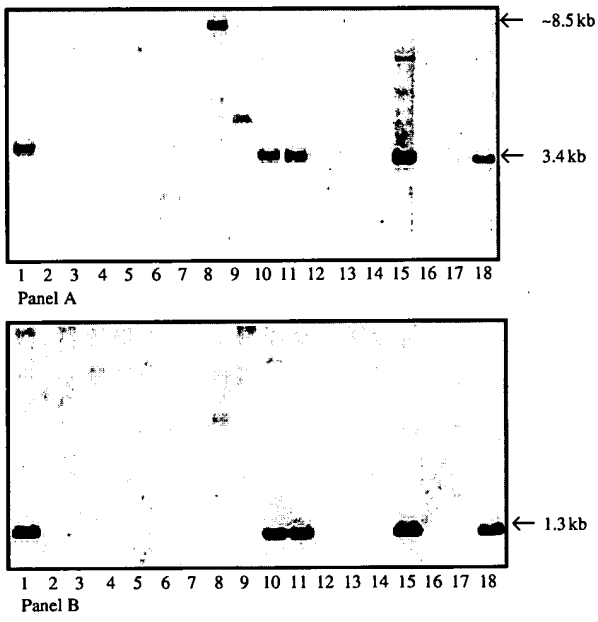

Figure 2 Southern hybridization analysis of tuberculous and nontuberculous mycobacterial DNAs probed with representative *Mycobacterium tuberculosis* genes. Chromosomal DNA was isolated from mycobacterial strains, subjected to restriction endonuclease digestion, separated by agarose gel electrophoresis, transferred to nylon membranes and hybridized with labelled DNA probes specific for each of the selected *M. tuberculosis* genes. DNA hybridization signals were detected by chemiluminescence. Results are shown for two representative *M. tuberculosis* genes. Panel A: Rv1271c, nonspecific for the *M. tuberculosis* complex; Panel B: Rv2253, specific for the *M. tuberculosis* complex. Lanes: (1) *M. tuberculosis*, strain W; (2) *Mycobacterium xenopi*; (3) *Mycobacterium ulcerans*; (4) *Mycobacterium scrofulaceum*; (5) *Mycobacterium phlei*; (6) *Mycobacterium microti*; (7) *Mycobacterium marinum*; (8) *Mycobacterium malmoense*; (9) *Mycobacterium kansasii*; (10) *Mycobacterium bovis* (clinical isolate); (11) *M. bovis* BCG substrain Japan; (12) *Mycobacterium intracellulare*; (13) *Mycobacterium haemophilum*; (14) *Mycobacterium fortuitum*; (15) *Mycobacterium africanum*; (16) *Mycobacterium avium* M-64; (17) *M. avium* and (18) *M. tuberculosis*, strain H_{37}Rv.

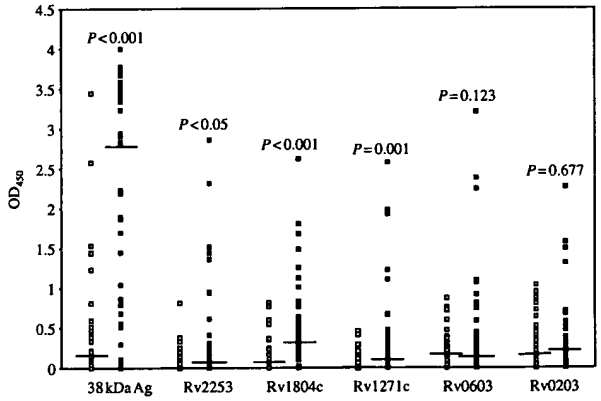

Figure 3 Serological reactivity of five novel secreted proteins and the 38 kDa antigen. Serological reactivity of five novel proteins (Rv0203, Rv0603, Rv1271c, Rv1804c and Rv2253) and a reference secreted antigen, the 38 kDa lipoprotein, was assessed by enzyme-linked immunosorbent assay using sera from patients having pulmonary tuberculosis (TB) ($n = 50$, ■) and control patients having lung disease other than TB ($n = 48$, □). Each data point represents one patient. Horizontal bars indicate median values. Comparisons of patient and control population were conducted by *t* test: differences in specific antibody levels between patient and control populations were examined using two-sided *t* tests and a significance level of 0.05.

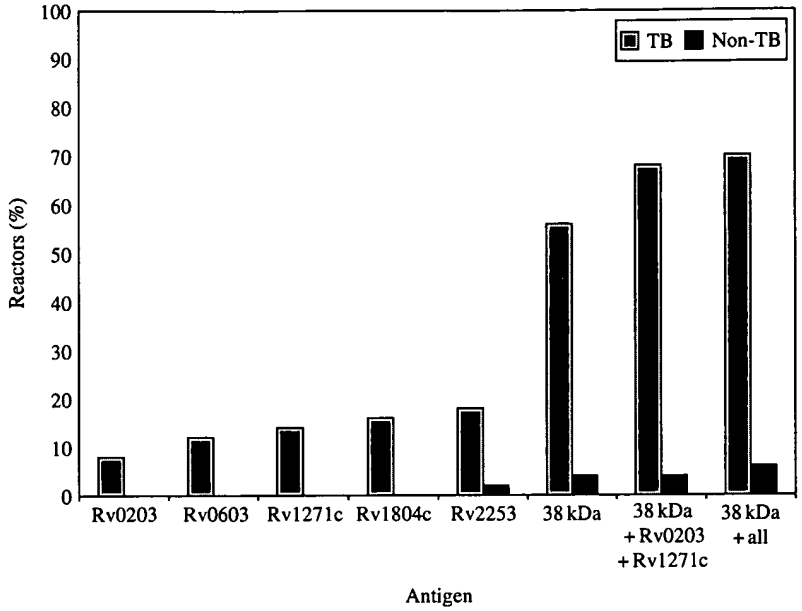

Figure 4 Diagnostic evaluation of single antigens and multiantigen combinations. The raw data shown in Fig. 3 were used to calculate a cutoff value [mean + 3 SD of enzyme-linked immunosorbent assay (ELISA) readings obtained with 48 nontuberculosis (non-TB) control sera] for each antigen and the proportion of positive test results in the TB and control patient populations obtained with single antigens and selected antigen combinations. The two rightmost data sets show the combined seroreactivity of the 38 kDa antigen plus the Rv0203 and Rv1271c antigens, and of the 38 kDa antigen plus all five novel secreted proteins, as indicated. Reactors (*y* axis) are subjects who gave ELISA readings equal to or above cutoff.

M. malmoense and gave a weak signal with *M. kansasii* (Fig. 2, panel A). Rv2253 was specific for the *M. tuberculosis* complex (Fig. 2, panel B).

Recombinant protein expression and purification

Selected genes were cloned in the pQE30 plasmid vector of *E. coli*, which expresses recombinant protein fused to an NH_2-terminal polyhistidine purification tag. Genes that did not express or expressed at a low level in this vector were cloned into a second plasmid, pET102, which expresses the recombinant protein fused to a COOH-terminal polyhistidine tag as well as to *E. coli* thioredoxin. Use of pET102 improved the yield of purified product by twofold to threefold for Rv1271c and Rv1804c (but not for Rv2389c) (data not shown). Of 10 genes selected, three (Rv0607, Rv1242 and Rv2389c) could not be cloned or expressed in *E. coli* (data not shown). The remaining seven genes (denoted by asterisks in Table 2) produced recombinant protein in *E. coli* that was purified to near-homogeneity by sequential chromatography.

Antibody responses in human TB

Serum levels of specific IgG antibodies to seven novel secreted proteins of *M. tuberculosis* were measured by enzyme-linked immunosorbent assay (ELISA) in 50 patients with pulmonary TB and 48 control patients having a pulmonary pathology other than TB. The 38 kDa lipoprotein [24], which is serodominant in humans [25, 26], was included in the analysis as a reference antigen. In pilot assays, Rv2290 gave high background readings with negative control sera, whereas Rv1813c consistently gave low readings with selected TB patient sera (data not shown). These two proteins were, therefore, excluded from further analysis. For two proteins (Rv0203 and Rv0603), we found no difference between serum antibody levels measured in untreated TB patients relative to control patients having lung disease other than TB. The remaining three proteins (Rv1271c, Rv1804c and Rv2253) elicited different antibody levels between untreated TB patients and the control group ($P < 0.05$ for Rv2253 and $P \leq 0.001$ for Rv1804c and Rv1271c) (Fig. 3). These data provide indirect evidence that Rv1271c, Rv1804c and Rv2253 proteins are produced by *M. tuberculosis* in patients. None of the novel proteins showed a level of reactivity comparable to that exhibited by the 38 kDa antigen (Fig. 4).

Because the 38 kDa antigen is a preferred reagent in multiantigen cocktails for TB serodiagnosis [25, 27], we determined whether the novel antigens had the potential to increase accuracy of a 38 kDa-based serodiagnostic test. Cutoff values (mean + 3 SD of ELISA readings obtained with control sera) were established to distinguish responders from nonresponders in both TB patients and control groups (Fig. 4). The 38 kDa lipoprotein was the most seroreactive antigen of the protein panel with TB patient sera (28/50, 56%). Reactivity of sera from TB patients ranged from 4/50 (8%) for Rv0203 to 9/50 (18%) for Rv2253. Two control sera gave a positive reaction with the 38 kDa antigen and one with Rv2253. No control sera reacted with Rv1804c, Rv1271c, Rv0603 or Rv0203. We do not know the reasons for high serum antibody levels to these two *M. tuberculosis*-complex-specific proteins in patients diagnosed as not having TB. In the previous work, we found that strong positive reactions ($OD_{450} > 2.5$) to the 38 kDa antigen in control sera were

often associated with history of past TB (G.V. Kanaujia *et al.*, submitted for publication; [28]). A review of clinical data for the seropositive control subjects in the present study, however, gave no conclusive information about their TB history. Further analysis showed that 18/50 (36%) of TB patient sera contained antibodies against at least one of the five novel secreted antigens and that 7/50 (14%) of TB patients had serum antibodies against at least one novel antigen, but they did not react against the 38 kDa antigen. We calculated that 34/50 TB patients (68%) had antibodies to 38 kDa, Rv0203 and Rv1271c combined. Combining the reactivity of all five novel proteins to that of the 38 kDa antigen increased the overall reactivity of TB patient group by only an additional 2% (Fig. 4).

In conclusion, we have identified six novel secreted proteins of *M. tuberculosis* that are specific for tuberculous mycobacteria. An additional four proteins may be present in nontuberculous mycobacteria with little medical or geographical significance (*M. kansasii* and *M. malmoense*). Our serological analysis provides evidence that at least three of these proteins are produced during active disease, because specific antibody levels differed between the TB patient group and the non-TB control group. Finally, the data suggest that adding Rv0203 and Rv1271c to the 38 kDa antigen may improve the sensitivity of a 38 kDa-antigen-based assay without decreasing specificity (no false positive result was obtained with Rv0203 and Rv1271c in the present work). The effect on diagnostic accuracy of adding novel secreted antigens identified in the present study to the 38 kDa lipoprotein will be further evaluated in independent populations, because antibody responses to particular *M. tuberculosis* antigens are likely affected by factors that include the host major histocompatibility complex phenotype, antigen immunodominance and antigen load [29, 30].

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